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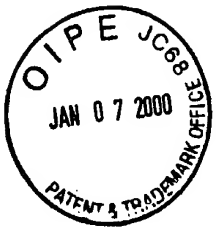

In re Patent Application of

ELMORE

Serial No. 08/981,087

Filed: May 27, 1998

Atty. Ref.: 124-688

Group: 1645

Examiner: Weatherspoon

For: TYPE F BOTULINUM TOXIN AND USE THEREOF

January 7, 2000

Assistant Commissioner for Patents
Washington, DC 20231

SUBMISSION OF PRIORITY DOCUMENT

Sir:

It is respectfully requested that this application be given the benefit of the foreign filing date under the provisions of 35 U.S.C. §119 of the following, a certified copy of which is submitted herewith:

Application No.	Country of Origin	Filed
9511909.5	UK	June 12, 1995

Respectfully submitted,

NIXON & VANDERHYE P.C.

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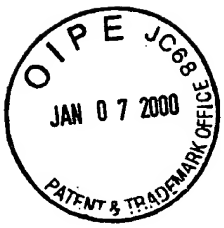

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Request for grant of a Patent

Form 1/77

Patents Act 1977

❶ Title of invention

1 Please give the title of the invention: VACCINE

❷ Applicant's details

❑ **First or only applicant**

2a If you are applying as a corporate body please give:

Corporate name: MICROBIOLOGICAL RESEARCH AUTHORITY

Country *(and State of incorporation, if appropriate)*: UNITED KINGDOM

2b If you are applying as an individual or one of a partnership please give in full:

Surname

Forenames

2c **In all cases**, please give the following details:

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UK postcode *(if applicable)*

Country: UNITED KINGDOM

ADP number *(if known)*: 06566160001

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Agent's address	100 GRAYS INN ROAD LONDON
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⑤ Claiming an arli r application date

5 Are you claiming that this application be treated as having been filed on the date of filing of an earlier application?

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8a Please fill in the number of sheets for each of the following types of document contained in this application.

Continuation sheets for this Patents Form 1/77	–		
Claim(s)	4	Description	13
Abstract	–	Drawing(s)	2

8b Which of the following documents also accompanies the application?

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Priority documents *(please state how many)*	–
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VACCINE

The present invention relates to type F botulinum toxin, to a fragment of type F botulinum neurotoxin, to production of the fragment by recombinant means and to a synthetic gene encoding the fragment. In particular, the invention relates to a novel polypeptide fragment capable of eliciting an immunological response that is protective against type F botulinum neurotoxin (BoNT/F) in man or animals and to a vaccine containing the fragment.

Botulinum neurotoxins (BoNT) are high molecular weight proteins (approx. 150,000 Da) which exert potent neuroparalytic effects on vertebrates. They are elaborated by anaerobic Gram-positive bacteria belonging to the genus *Clostridium*. The majority of clostridia which produce BoNT are classified as *Clostridium botulinum*. In recent years, however, isolates which resemble *Clostridium barati* and *Clostridium butyricum* have been shown to produce BoNT. On the basis of antigenicity, BoNT has been subdivided into seven distinct types, designated A to G. All seven neurotoxins (BoNT/A to BoNT/G) are synthesised as a single-chain 150,000 Da molecule which subsequently become nicked to the more potent di-chain form, composed of a heavy (H) chain (approx. 100,000 Da) and a light (L) chain (50,000 Da) polypeptide linked by at least one disulphide bridge.

The action of BoNT involves three distinct phases. In the first phase the toxins become bound to acceptors on the external surface of the targeted neural cells. This is followed by an energy dependent internalisation step in which the toxin, or part of it, enters the cell. Thereafter the active moiety of the toxin causes nerve cell dysfunction by blocking the intracellular release of the neurotransmitter, acetylcholine, at the nerve periphery, causing flaccid paralysis. The L chain possesses the catalytic activity responsible for cell poisoning and the H chain delivers this moiety to the cell cytoplasm by mediating binding of the toxin to the cell and subsequent internalisation.

The entire amino acid sequences of all 7 BoNTs are now known [Minton, N.P. (1995). Current Topics in Microbiology and Immunology 195: 161-187], revealing them to be surprisingly divergent in their primary amino acid sequences. Thus, sequence identity amongst the different serotypes generally does not exceed 40%, with those areas of homology localised to discrete domains which are interspersed with amino acid tracts exhibiting little overall similarity. Between the different L chains (average size 439), 63 amino acids are absolutely conserved. Throughout the H chains (average size 843) 97 amino acids are identical. The most notable areas of conservation include:- the two cysteine residues involved in the disulphide bond formation between the L and H chain; the histidine rich motif within the L chain associated with metalloprotease activity; and a highly conserved PYI/VXALN-motif found adjacent to regions identified as possessing membrane spanning potential. The most notable tract of sequence divergence amongst toxins is localised to the COOH-terminus of their respective H chains (amino acid 1124 onwards of BoNT/A). This would appear to be consistent with the notion that this domain is involved in neurotoxin binding and that different toxins target different acceptors on neural cell surfaces.

The effectiveness of modern food-preserving processes in Western countries has made outbreaks of botulism extremely rare. The frequent use of *C.botulinum* as a test organism in the food industry, and the growing use of the toxin by neurobiochemists, has, however, increased the need for human vaccines. The formulation of these vaccines has changed little since the early 1950s: partially purified preparations of the neurotoxins are toxoided by formaldehyde treatment and absorbed onto precipitated aluminium salts. Using such methodology, polyvalent vaccines (against ABCDE or ABEF) for human immunisation are currently available. Such vaccines suffer from the drawback of low immune response and considerable batch to batch variation due to the high proportion (60-90%) of contaminating proteins in toxoid preparations. Recent work has therefore concentrated on the development of procedures for the purification of toxins to near-homogeneity. The use of purified toxins in the production of vaccines, however, suffers from the drawbacks, first, of having to produce them under high

containment and, secondly, of requiring the presence of low levels of formaldehyde to prevent possible reversion of the toxoid to the active state.

Production of subunit vaccines against other organisms/ toxins have been investigated by a number of laboratories. This work has focused on the best known toxin subtypes, namely A and B, leading to new vaccines giving specific immunity against toxins of type A or B. Each new vaccine, however, may not give protection against other toxin subtypes.

Recombinant production of vaccine components has brought great advances in vaccine purity and volume of production but nevertheless requires a large number of steps to recover purified vaccine components from the host cell.

It is an object of this invention to produce a vaccine against a type F botulinum toxin. It is another object to simplify vaccine manufacture. A further object is to improve production of botulinum toxin vaccines. A still further object of the invention is to overcome or at least mitigate problems and/or limitations in existing vaccines and methods of production.

According to a first aspect of the invention there is provided a polypeptide free of botulinum toxin activity which induces protective immunity to a type F botulinum toxin.

A first aspect of the invention alternatively provides a polypeptide characterized in that it:-

(a) is free of botulinum toxin activity, and

(b) is capable of eliciting, in a mammal, an immunological response that is protective against type F botulinum toxin.

The term "protective" used in conjunction with "immunity" and "immunological response" is used to indicate an increased ability to survive challenge by active

botulinum toxin. This increase is typically mediated by an increased titre of antibodies to the toxin or an increased ability to produce antibodies to the toxin upon challenge with toxin. The term is not intended to indicate absolute protection against any amount of toxin.

The invention thus offers specific protection against a type F botulinum toxin, protection that has hitherto been unavailable.

In a particular embodiment the present invention provides a peptide or peptide conjugate comprising the amino acid sequence of the *C. botulinum* strain Langeland BoNT/F from amino acids 848 to 1278, but lacking the amino acid sequences of the L chain and H_N epitopes necessary for metalloprotease activity and toxin internalisation (found between amino acids 1 to 439 and 440 to 847, respectively); the peptide is capable of inducing an immune response protective against BoNT/F when administered to humans or other animals.

In a more particular embodiment the peptides of the invention consist of only the sequence of amino acids from 848 to 1278 of the amino acid sequence of BoNT/F of the *Clostridium botulinum* strain Langeland, or of that sequence in the form of a fusion peptide with an amino acid sequence other than amino acids 1 to 847 of BoNT/F, or in the form of a conjugate with an agent having other desired effect. The term 'other amino acid sequence' will be understood by the person skilled in the art to include complete proteins as well as relatively short amino acid sequences as appropriate to the needs of the user. Optionally, the other sequence is a non-*C. botulinum* antigenic protein which is included fused to the aforesaid sequence for the purpose of providing other immunity or labelling, or for expression purposes.

In another embodiment of the invention the polypeptide comprises a fragment or a derivative of a type F botulinum neurotoxin, or, in particular, consists of said fragment or said derivative.

A polypeptide according to a further embodiment comprises a fusion protein of:-

(a) amino acids 848 to 1278 of a type F botulinum neurotoxin, with

(b) a purification moiety.

It is preferred that the purification moiety is adapted to bind to an affinity chromatography column. A particular purification moiety comprises from 50 to 500 amino acids. In a specific embodiment the fusion protein comprises maltose-binding protein. This fusion protein is particularly suitable for purification using an affinity chromatography column.

In further specific embodiments of the invention the fragment is selected from:-

(a) amino acids 848-1278 of a type F botulinum toxin,

(b) amino acids 848-991 of a type F botulinum toxin,

(c) amino acids 992-1135 of a type F botulinum toxin, and

(d) amino acids 1136-1278 of a type F botulinum toxin.

The invention also relates to a toxin derivative, being synthetic fragments of a type F botulinum toxin linked together in repeated sections. In an embodiment said derivative comprises a dimer of the fragments specified above.

According to a second aspect the invention provides a vaccine against a botulinum toxin, comprising a polypeptide of the first aspect and a pharmaceutically acceptable carrier.

Suitable carriers are known to a person of skill in the for preparation of the vaccine. In an embodiment hereinafter described the carrier includes Freund's adjuvant. Another suitable carrier component is precipitated alum salts.

In a third aspect of the present invention there is provided recombinant DNA encoding peptides of the invention. Such recombinant DNA is conveniently provided by PCR amplification of the DNA coding for the desired sequence, eg., BoNT/$F_{848-1278}$, using primers targeted at respective ends of the double stranded

sequence. Optionally the template sequence used in PCR represents the natural clostridial gene. In the preferred form of the aspect, however, the sequence used is a synthetic sequence encoding the same amino acids as the natural clostridial protein but in which codon usage has been altered. It is preferred that the synthetic gene has a GC content of at least 40%, preferably at least 45% and most preferably at least 50%.

In the case of such a synthetic sequence, insertion into the chosen expression plasmid is achieved, in one embodiment of the invention, through the use of incorporated appropriate restriction endonuclease recognition sites positioned at the extremities of the DNA fragment during its construction.

By whatever means the recombinant DNA encoding the BoNT/F peptide is generated, it is ligated into a suitable expression vector at which stage genetic fusion to a desired fusion peptide encoding sequence occurs, if desired, and the resultant vector introduced into a suitable cell line, eg., *E. coli* or a yeast such as *Pichia pastoris*. A cell line producing the desired product is selected through established procedures, eg., Western Blotting, or ELISA.

Fourth and fifth aspects of the invention provide respectively, a plasmid vector incorporating the DNA of the third aspect and a cell line comprising the plasmid and expressing the DNA.

In a sixth aspect of the invention there is provided a method of producing a polypeptide of the first aspect comprising the steps of:-

(a) expressing in a host cell a DNA encoding a fusion protein, said protein being a fusion of (i) a fragment of a type F botulinum toxin, and (ii) a purification moiety adapted to bind to an affinity chromatography column,

(b) obtaining from said host cell an extract comprising the fusion protein, and

(c) purifying the fusion protein using an affinity chromatography column.

In use of an embodiment of the invention the fusion protein is removed from the column by elution with a substrate. The method optionally includes cleaving the fusion protein and retaining the toxin fragment.

By this method the invention enables a preparation of a botulinum toxin type F fragment free of contamination by other clostridial proteins, these latter frequently contaminating prior art preparations derived from cultures of clostridium bacteria.

The fusion protein or toxin fragment obtained is typically in a substantially pure form and suitable for incorporation into a vaccine or other pharmaceutical composition in a few simple steps.

It should be noted that the creation of certain fusion proteins comprising the BoNT/F-derived peptide is useful in the initial isolation of the latter, following which cleavage is optionally employed to purify the BoNT/F-related peptide. Where codons are added at the 5'-end of the BoNT/F-encoding DNA to aid in translation, these amino acids are optionally retained at the NH_2- terminal end of the final peptide, eg., those used to bring about secretion of the peptide or more simply the addition of an NH_2-terminal methionine to initiate translation.

A seventh aspect of the invention provides a method of making a pharmaceutical composition comprising:-

(a) expressing in a host cell a DNA encoding a fusion protein, said protein being a fusion of (i) a botulinum toxin or a fragment thereof, and (ii)a purification moiety adapted to bind to an affinity chromatography column,

(b) obtaining from said host cell an extract comprising the fusion protein,

(c) purifying the fusion protein using an affinity chromatography column,

(d) incorporating the purified fusion protein into a pharmaceutical composition.

The method is suitable for expression both of active and inactive toxin fragments, though it is preferred that said fragment is free of toxin activity.

The purification moiety typically comprises 50 to 500 amino acids, is water soluble and binds to an affinity chromatography column.

The inventors have found that a fusion protein retaining the purification moiety is of advantage in producing for example a vaccine against a type F botulinum toxin. Vaccinating activity is found in the fusion protein, so the purification protein does not need to be removed prior to vaccine manufacture, thus simplifying the manufacturing process. It is preferred that the purification protein is a globular, water soluble protein that binds to and can be purified using an affinity chromatography column. It is further preferred that the purification protein is highly immunogenic. Thus, a particularly preferred fusion protein comprises a fragment of a botulinum toxin, an immunogenic region and a purification end adapted to bind to an affinity chromatography column.

The term immunogenic region is used above to describe a sequence of amino acids in a protein that is known to elicit stimulation of the immune system in humans or other animals. Examples of such an immunogenic region include keyhole limpet haemocyanin.

Further aspects of the invention provide a pharmaceutical containing the fusion protein, methods of vaccinating mammals using the vaccines and compositions of the invention and antisera raised against the polypeptides, vaccines and compositions of the invention.

There now follows description of specific embodiments of the invention, illustrated by drawings in which:-

Figure 1: shows the three major domains of a BoNT toxin. The numbers refer to the positions of the amino acids flanking these three domains in BoNT/F of

C.botulinum strain Langeland;

Figure 2: shows the amino acid sequence of the H_c fragment of BoNT/F from *Clostridium botulinum* type F strain Langeland between amino acid position 848 and 1278;

Figure 3: shows the nucleotide sequence of the region of the BoNT/F gene from *Clostridium botulinum* type F strain Langeland encoding the H_c fragment;

Figure 4: shows a schematic representation of how synthetic gene blocks were assembled by PCR;

Figure 5: shows a synthetic DNA sequence encoding the BoNT/F H_c fragment which uses codons which are used most frequently in highly expressed genes of E. coli. The codon corresponding to BoNT/F Ser_{848} begins at nucleotide position 12. It is proceeded by a codon specifying a NH_2-terminal methionine codon and restriction sites for *Nde*I and *Bam*HI. The codon for Asn_{1278} begins at nucleotide position 1302, and is followed by a translational stop codon (nt 1305-1308) and a restriction site for *Xba*I;

Figure 6: shows an example of a recombinant plasmid (pFHC206) made in which the synthetic DNA fragment in Figure 5 is inserted into the expression plasmid pMal-C2; and

Figure 7: shows antibody titres against BoNT/F obtained in mice immunised with MBP-BoNT/$F_{848-1278}$ recombinant protein.

EXAMPLES

Generation of a synthetic DNA fragment encoding H_c of BoNT/F which makes use of codons utilised by highly expressed E. coli genes

A synthetic sequence encoding BoNT/$F_{848-1278}$ was designed by reverse translation of the BoNT/F amino acid sequence using the REVTRANS programme of DNASTAR Inc (Madison, USA). The codon code used was the "strongly expressed E. coli backtranslation code" (SECOLI.RTC). To facilitate the construction, a number of changes were then made to introduce restriction enzyme recognition sites at strategic points along the length of the fragment, including unique flanking proximal sites for *Bam*HI and *Nde*I a distal flanking site for *Xba*I and internal sites for *Hpa*I, *Mlu*I and *Sph*I. The gene was then constructed from overlapping 100 mer oligonucleotides by a procedure essentially as described elsewhere [Sandhu *et al* (1992) Biotechniques 12:14-16].

Briefly, the gene was constructed as 4 individual blocks (A, B, C and D), each of approximately 350 bp in size. Each block was assembled from 4 x 100 mer alternating oligonucleotides which overlapped with each other by 20 nucleotides. These 4 oligonucleotides were used in a PCR to generate a composite c.350 bp double-stranded DNA fragment, which was subsequently amplified using 20 mer flanking primers. The amplified fragments of each block were then cloned directly into plasmid pCRII (Invitrogen Corp). The flanking primers of all 4 blocks were designed to include restriction enzyme sites which would allow their subsequent assembly into a contiguous fragment. Thus, block A was flanked by *Bam*HI (5') and *Hpa*I (3'), block B by *Hpa*I (5') and *Mlu*I (3'), block C by *Mlu*I (5') and *Sph*I (3'), and block D by *Sph*I (5') and *Xba*I (3'). Each block was, therefore, released from their respective pCRII-derived recombinant plasmid by cleavage with the appropriate enzyme and the isolated fragments ligated to pMTL23 [Chambers *et al* (1988). Gene 68:139-149] plasmid DNA which had been cleaved with *Bam*HI and *Xba*I. A clone was then selected in which all 4 blocks had been inserted in the expected order. This was confirmed by nucleotide sequencing using routine methods [Maniatis *et al*. (1989). Molecular Cloning a Laboratory Manual. Cold Spring Harbor Laboratory Press], and the plasmid obtained designed pFHC23.

Generation of a H_C peptide (848 to 1278) of BoNT/F of C. botulinum strain Langeland

A candidate vaccine against the BoNT/F of *C. botulinum* was produced by expressing the fragment of the synthetic gene encoding the H_c fragment, amino acids 848 to 1278. This DNA fragment was isolated from plasmid pFHC23 as an approximately 1.3 kb *Bam*HI-*Xho*I restriction fragment and inserted between the unique *Bam*HI and *Sal*I sites of pUC9 [Vieira and Messing (1982). Gene 19: 259-268], generating the plasmid pFHC29. The insert was then reisolated from pFHC29 as an *Eco*RI-*Xba*I fragment and inserted between the equivalent sites of the commercially available expression vector pMal-c2 (New England Biolabs), to yield the final plasmid pFHC206. The resultant plasmid expressed BoNT/$F_{848-1278}$ as a fusion protein with the vector encoded maltose binding protein (MBP).

Fusion protein product (MBP-BoNT/$F_{848-1278}$) was prepared from the cell line containing pFHC206 in the following manner. *E. coli* containing pFHC206 was cultivated in 1 litre of media (M9, supplemented with 0.8M sorbitol, 0.5% casamino acids, 50 μg/ml ampicillin), shaking (200 rpm) at 37°C until an OD_{600} of 1.0 was achieved. At this point IPTG was added at a final concentration of 1 mM and shaking continued at 27°C for a further 4 hour. Cells were harvested by centrifugation (5000 x *g*) and resuspended in 20 ml of lysis buffer (Protein Fusion and Purification System, New England Biolabs) and cells disrupted by sonication. Lysate was applied to a GPC column containing 180 ml of Sephacel S100, and the protein in the void fraction collected. MBP-BoNT/F $H_{848-1278}$ fusion protein in this fraction was then allowed to adsorb at room temperature to a 4-6 ml volume of Amylose resin (New England Laboratories) over a 3 hour period with gentle shaking (10 rpm). Recombinant fusion protein was then eluted in buffer (0.01M Tris HCl, pH 7.0) containing 5 mM maltose. Eluted protein was concentrated using an Amicon PM30 membrane filter.

Toxicity of candidate vaccine

The toxicity of the candidate vaccine fusion peptide was determined by intraperitoneal inoculation of 25 μg amounts of the total recombinant MBP-

BoNT/$F_{848-1278}$ protein into groups of 4 mice. The candidate vaccine was well tolerated and mice showed no signs of acute or chronic toxicity up to 2 weeks post inoculation.

Antibody responses to candidate vaccines

The candidate vaccine was administered to groups of 4 mice by intraperitoneal inoculation in complete Freund's adjuvant, and a booster inoculation given on 3 further occasions at two week intervals. Antibody response against purified *C. botulinum* strain Langeland BoNT/F was monitored by ELISA (Fig 7).

Protection against toxin challenge

Animals which were immunised with MBP-BoNT/$F_{848-1278}$ fusion protein were subjected to an intraperitoneal challenge with various doses of purified *C. botulinum* strain Langeland BoNT/F. At doses of 12 LD_{50} and above, all the control, unimmunised mice succumbed within 24 hour. All immunised groups of mice survived challenges of up to 2.4×10^4 LD_{50} . One of the immunised mice which had survived an initial challenge of 1.8, LD_{50} was subsequently shown to be immune to a further challenge of 10^6 LD_{50} .

TABLE 1: Protection against challenge with *C. botulinum* strain Langeland BoNT/F afforded by the MBP-BoNT/$F_{848-1278}$ fusion protein vaccine. A total of 4 X 25 μg intraperitoneal doses of antigén mixed with adjuvant were given to groups of 4 mice at 14 day intervals. After 50 days mice were subjected to intraperitoneal challenges of varying levels of purified BoNT/F, (isolated from *C. botulinum* strain Langeland), and deaths recorded up to 4 days.

Challenge Dose (LD_{50})	Mortality/Total Animals	
	Control Animals	Immunised Animals
2.4×10^4	4/4	0/4
3.6×10^3	4/4	0/4
5.4×10^2	4/4	0/4
81	4/4	0/4
12	4/4	0/4
1.8	2/4	0/4[a]

[a] = one of the surviving individuals from this group was subsequently shown to be protected against a BoNT/F challenge equivalent to 10^6 LD_{50}.

This invention provides a fragment (such as amino acids 848-1278) of BoNT/F isolated from *C. botulinum* strain Langeland for use as a vaccine. The fragment retains its immunogenic properties while still fused with MBP, dispensing with the need for an additional purification step. The recombinant fusion protein appears to be non-toxic in mice at doses up to 25μg. Repeated doses produced a significant antibody response which protects animals against BoNT/F challenge. As a vaccine it offers several advantages over neurotoxin toxoided by formaldehyde treatment. Most notably, it may be prepared more easily and, due to the absence of active toxin, at a lower level of containment. The absence of other contaminating *C.botulinum* proteins and partially toxoided materials also make it inherently safer for vaccine application and less reactogenic.

CLAIMS

1. A polypeptide free of botulinum toxin activity which induces protective immunity to a type F botulinum toxin.

2. A polypeptide characterized in that it:-
   (a) is free of botulinum toxin activity, and
   (b) is capable of eliciting, in a mammal, an immunological response that is protective against type F botulinum toxin.

3. A polypeptide according to Claim 1 or 2 comprising a fragment or a derivative of a type F botulinum neurotoxin.

4. A polypeptide according to Claim 3 consisting of said fragment or said derivative.

5. A polypeptide according to Claim 3 or 4 comprising a fusion protein of:-
   (a) amino acids 848 to 1278 of a type F botulinum neurotoxin, with
   (b) a purification moiety.

6. A polypeptide according to Claim 5 wherein the purification moiety is adapted to bind to an affinity chromatography column.

7. A polypeptide according to Claim 5 or 6 wherein the purification moiety comprises from 50 to 500 amino acids.

8. A polypeptide according to any of Claims 5-7 wherein the fusion protein comprises maltose-binding protein.

9. A polypeptide according to any of Claims 3-8 wherein said fragment is selected from:-
   (a) amino acids 848-1278 of a type F botulinum toxin,

(b) amino acids 848-991 of a type F botulinum toxin,

(c) amino acids 992-1135 of a type F botulinum toxin, and

(d) amino acids 1136-1278 of a type F botulinum toxin.

10. A polypeptide according to any of Claims 3-8 wherein said derivative comprises a dimer of the fragment according to any of (a)-(d) of Claim 9.

11. A vaccine comprising a pharmaceutically acceptable carrier and a polypeptide according to any of Claims 1-10.

12. A recombinant DNA encoding a polypeptide according to any of Claims 1-11.

13. A method of producing a polypeptide according to any of Claims 1-11 comprising the steps of:-

(a) expressing in a host cell a DNA encoding a fusion protein, said protein being a fusion of (i) a fragment of a type F botulinum toxin, and (ii) a purification moiety adapted to bind to an affinity chromatography column,

(b) obtaining from said host cell an extract comprising the fusion protein, and

(c) purifying the fusion protein using an affinity chromatography column.

14. A method according to Claim 13 wherein the fusion protein is removed from the column by elution with a substrate.

15. A method according to Claim 13 or 14 further comprising cleaving the fusion protein and retaining the toxin fragment.

16. A method according to any of Claims 13-15 wherein the purified fusion protein is free of other clostridial proteins.

17. A method of making a pharmaceutical composition comprising:-

(a) expressing in a host cell a DNA encoding a fusion protein, said protein being a fusion of (i) a botulinum toxin or a fragment thereof, ~~and (ii)~~ a purification moiety adapted to bind to an affinity chromatography column,

(b) obtaining from said host cell an extract comprising the fusion protein,

(c) purifying the fusion protein using an affinity chromatography column,

(d) incorporating the purified fusion protein into a pharmaceutical composition.

18. A method according to Claim 17 wherein said fragment is free of toxin activity.

19. A method according to Claim 17 or 18 wherein said purification moiety comprises 50 to 500 amino acids and binds to an affinity chromatography column.

20. A pharmaceutical composition comprising:-

(a) a fusion protein, said protein being a fusion of (i) a botulinum toxin or a fragment thereof, and (ii) a purification moiety; and

(b) a pharmaceutically acceptable carrier.

21. A pharmaceutical composition according to Claim 20 wherein said fusion protein comprises a fragment of botulinum toxin free of toxin activity.

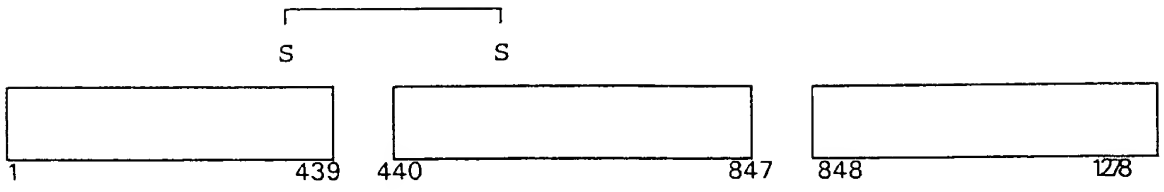
22. A method of vaccinating a mammal against a botulinum toxin, comprising administering to said mammal a vaccine according to Claim 11.

23. A method of vaccinating a mammal against a botulinum toxin, comprising administering to said mammal a pharmaceutical composition according to Claim 21.

24. Antisera raised to a polypeptide according to any of Claims 1-10.

25. Antisera raised to a vaccine according to Claim 11.

26. Antisera raised to a pharmaceutical composition according to Claim 21.



L Chain
(50 kDa)

H_N- fragment
(50 kDa)

H_C-fragment
(50 kDa)

H Chain (100kDa)

FIG. 1

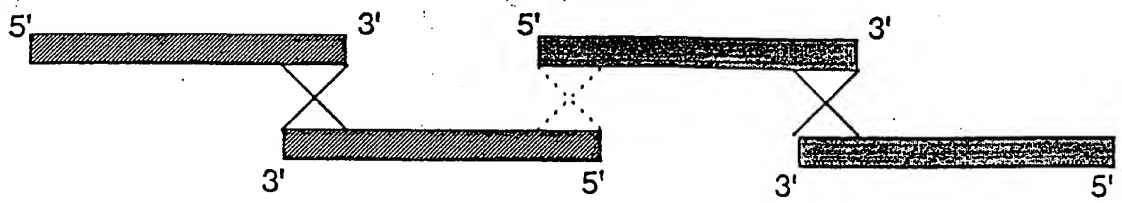
```
  1 SYTNDKILILYFNKLYKKIKDNSILDMRYENNKFIDISGYGSNISINGDVYIYSTNRNQF  60

 61 GIYSSKPSEVNIAQNNDIIYNGRYQNFSISFWVRIPKYFNKVNLNNEYTIIDCIRNNNSG 120

121 WKISLNYNKIIWTLQDTAGNNQKLVFNYTQMISISDYINKWIFVTITNNRLGNSRIYING 180

181 NLIDEKSISNLGDIHVSDNILFKIVGCNDTRYVGIRYFKVFDTELGKTEIETLYSDEPDP 240

241 SILKDFWGNYLLYNKRYYLLNLLRTDKSITQNSNFLNINQQRGVYQKPNIFSNTRLYTGV 300

301 EVIIRKNGSTDISNTDNFVRKNDLAYINVVDRDVEYRLYADISIAKPEKIIKLIRTSNSN 360

361 NSLGQIIVMDSIGNNCTMNFQNNNGGNIGLLGFHSNNLVASSWYYNNIRKNTSSNGCFWS 420

421 FISKEHGWQEN                                                   431
```

FIG. 2

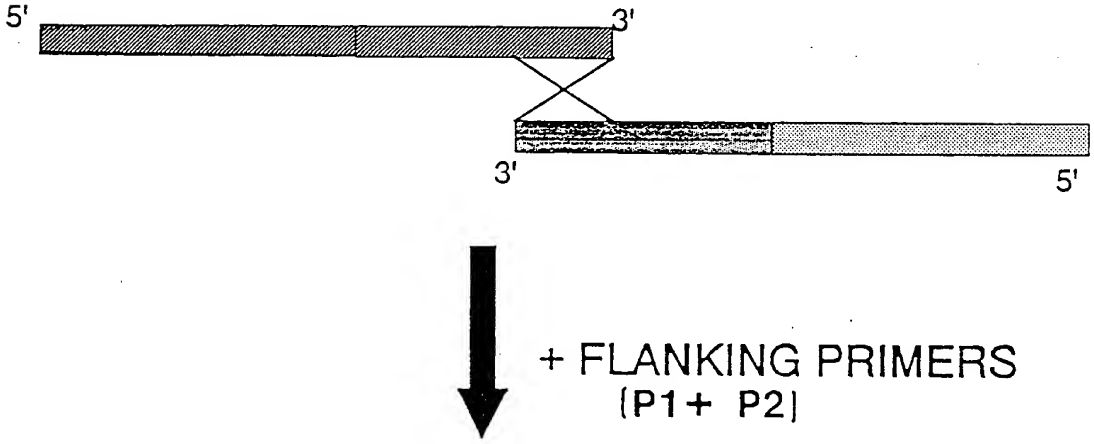
```
   1 TCATATACTAATGATAAAATTCTAATTTTATATTTTAATAAATTATATAAAAAAATTAAA  60
  61 GATAACTCTATTTTAGATATGCGATATGAAAATAATAAATTTATAGATATCTCTGGATAT 120
 121 GGTTCAAATATAAGCATTAATGGAGATGTATATATTTATTCAACAAATAGAAATCAATTT 180
 181 GGAATATATAGTAGTAAGCCTAGTGAAGTTAATATAGCTCAAAATAATGATATTATATAC 240
 241 AATGGTAGATATCAAAATTTTAGTATTAGTTTCTGGGTAAGGATTCCTAAATACTTCAAT 300
 301 AAAGTGAATCTTAATAATGAATATACTATAATAGATTGTATAAGGAATAATAATTCAGGA 360
 361 TGGAAAATATCACTTAATTATAATAAAATAATTTGGACTTTACAAGATACTGCTGGAAAT 420
 421 AATCAAAAACTAGTTTTTAATTATACACAAATGATTAGTATATCTGATTATATAAATAAA 480
 481 TGGATTTTTGTAACTATTACTAATAATAGATTAGGCAATTCTAGAATTTACATCAATGGA 540
 541 AATTTAATAGATGAAAAATCAATTTCGAATTTAGGTGATATTCATGTTAGTGATAATATA 600
 601 TTATTTAAAATTGTTGGTTGTAATGATACAAGATATGTTGGTATAAGATATTTTAAAGTT 660
 661 TTTGATACGGAATTAGGTAAAACAGAAATTGAGACTTTATATAGTGATGAGCCAGATCCA 720
 721 AGTATCTTAAAAGACTTTTGGGGAAATTATTTGTTATATAATAAAAGATATTATTTATTG 780
 781 AATTTACTAAGAACAGATAAGTCTATTACTCAGAATTCAAACTTTCTAAATATTAATCAA 840
 841 CAAAGAGGTGTTTATCAGAAACCAAATATTTTTTCCAACACTAGATTATATACAGGAGTA 900
 901 GAAGTTATTATAAGAAAAAATGGATCTACAGATATATCTAATACAGATAATTTTGTTAGA 960
 961 AAAAATGATCTGGCATATATTAATGTAGTAGATCGTGATGTAGAATATCGGCTATATGCT 1020
1021 GATATATCAATTGCAAAACCAGAGAAAATAATAAAATTAATAAGAACATCTAATTCAAAC 1080
1081 AATAGCTTAGGTCAAATTATAGTTATGGATTCAATAGGAAATAATTGCACAATGAATTTT 1140
1141 CAAAACAATAATGGGGGCAATATAGGATTACTAGGTTTTCATTCAAATAATTTGGTTGCT 1200
1201 AGTAGTTGGTATTATAACAATATACGAAAAAATACTAGCAGTAATGGATGCTTTTGGAGT 1260
1261 TTTATTTCTAAAGAGCATGGATGGCAAGAAAAC                              1293
```

FIG. 3

FIRST AMPLIFICATION, FIRST ROUND



FIRST AMPLIFICATION, SECOND ROUND



SECOND AMPLIFICATION

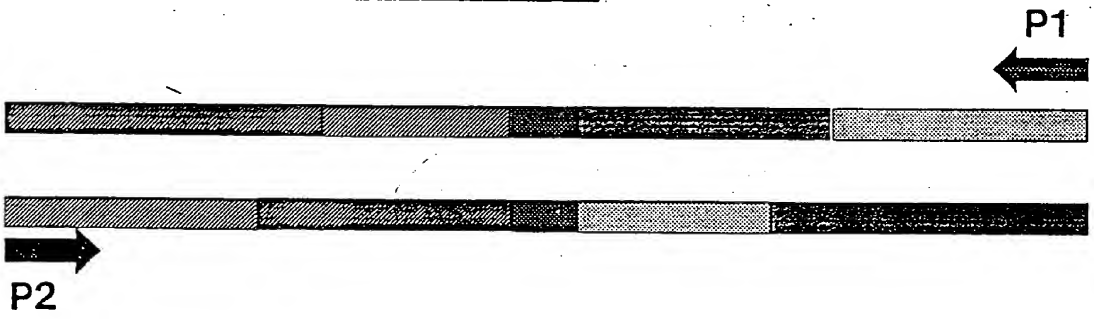
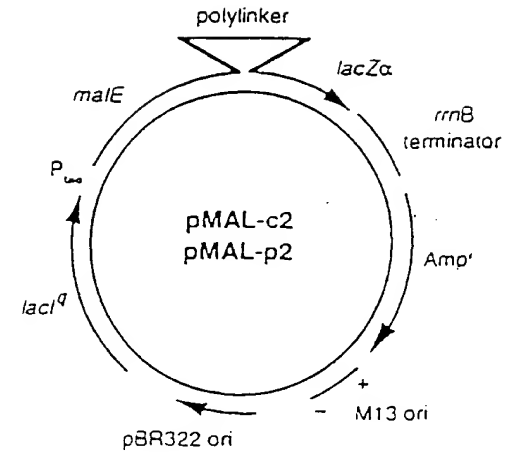


FIGURE 4

```
   1 GGATCCATATGTCTTACACTAACGACAAAATCCTGATCCTGTACTTCAACAAACTGTACA  60
  61 AAAAAATCAAAGACAACTCTATCCTGGACATGCGTTACGAAAACAACAAATTCATCGACA 120
 121 TCTCTGGCTATGGTTCTAACATCTCTATCAACGGTGACGTCTACATCTACTCTACTAACC 180
 181 GCAACCAGTTCGGTATCTACTCTTCTAAACCGTCTGAAGTAAACATCGCTCAGAACAACG 240
 241 ACATCATCTACAACGGTCGTTACCAGAACTTCTCTATCTCTTTCTGGGTTCGTATCCCGA 300
 301 AATACTTCAACAAAGTTAACCTGAACAACGAATACACTATCATCGACTGCATCCGTAACA 360
 361 ACAACTCTGGTTGGAAAATCTCTCTGAACTACAACAAAATCATCTGGACTCTGCAGGACA 420
 421 CTGCTGGTAACAACCAGAAACTGGTTTTCAACTACACTCAGATGATCTCTATCTCTGACT 480
 481 ACATTAATAAATGGATCTTCGTTACTATCACTAACAACCGTCTGGGTAACTCTCGTATCT 540
 541 ACATCAACGGTAACCTGATCGATGAAAAATCTATCTCTAACCTGGGTGACATCCACGTTT 600
 601 CTGACAACATCCTGTTCAAAATCGTTGGTTGCAACGACACGCGTTACGTTGGTATCCGTT 660
 661 ACTTCAAAGTTTTCGACACTGAACTGGGTAAAACTGAAATCGAAACTCTGTACTCTGACG 720
 721 AACCGGACCCGTCTATCCTGAAAGACTTCTGGGGTAACTACCTGCTGTACAACAAACGTT 780
 781 ACTACCTGCTGAACCTGCTCCGGACTGACAAATCTATCACTCAGAACTCTAACTTCCTGA 840
 841 ACATCAACCAGCAGCGTGGTGTTTATCAGAAACCTAATATCTTCTCTAACACTCGTCTGT 900
 901 ACACTGGTGTTGAAGTTATCATCCGTAAAAACGGTTCTACTGACATCTCTAACACTGACA 960
 961 ACTTCGTACGTAAAAACGACCTGGCTTACATCAACGTTGTTGACCGTGACGTTGAATACC 1020
1021 GTCTGTACGCTGACATCTCTATCGCTAAACCGGAAAAAATCATCAAACTGATCCGTACTT 1080
1081 CTAACTCTAACAACTCTCTGGGTCAGATCATCGTTATGGACTCGATCGGTAACAACTGCA 1140
1141 CTATGAACTTCCAGAACAACAACGGTGGTAACATCGGTCTGCTGGGTTTCCACTCTAACA 1200
1201 ACCTGGTTGCTTCTTCTTGGTACTACAACAACATCCGTAAAAACACTTCTTCTAACGGTT 1260
1261 GCTTCTGGTCTTTCATCTCTAAAGAACACGGTTGGCAGGAAAACTAATCTAGA         1313
```

FIG. 5

pMAL™-c2, -p2:



pMAL™-c2, -p2 polylinker:

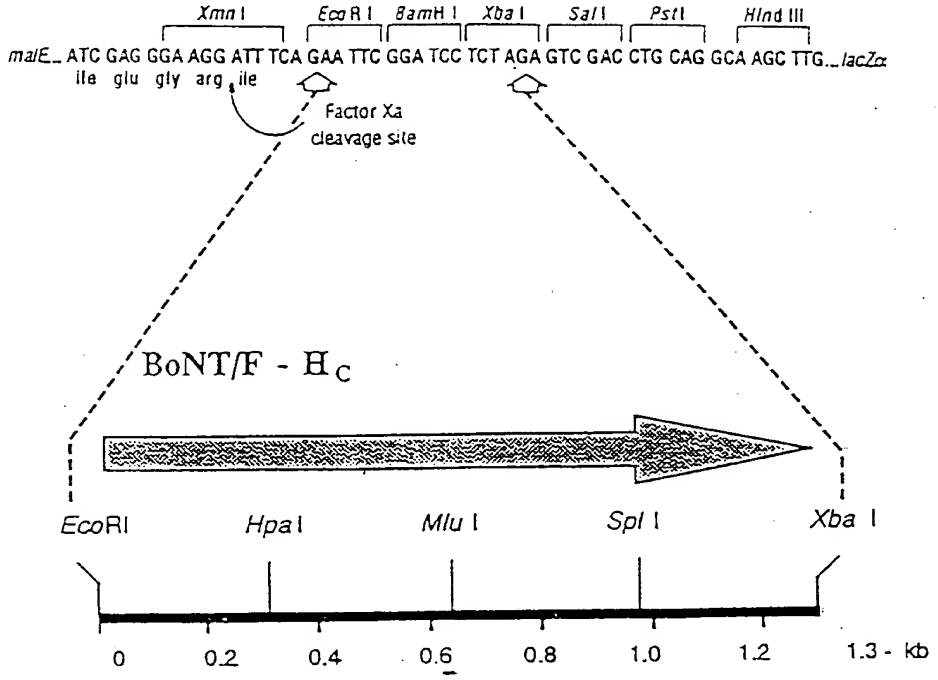
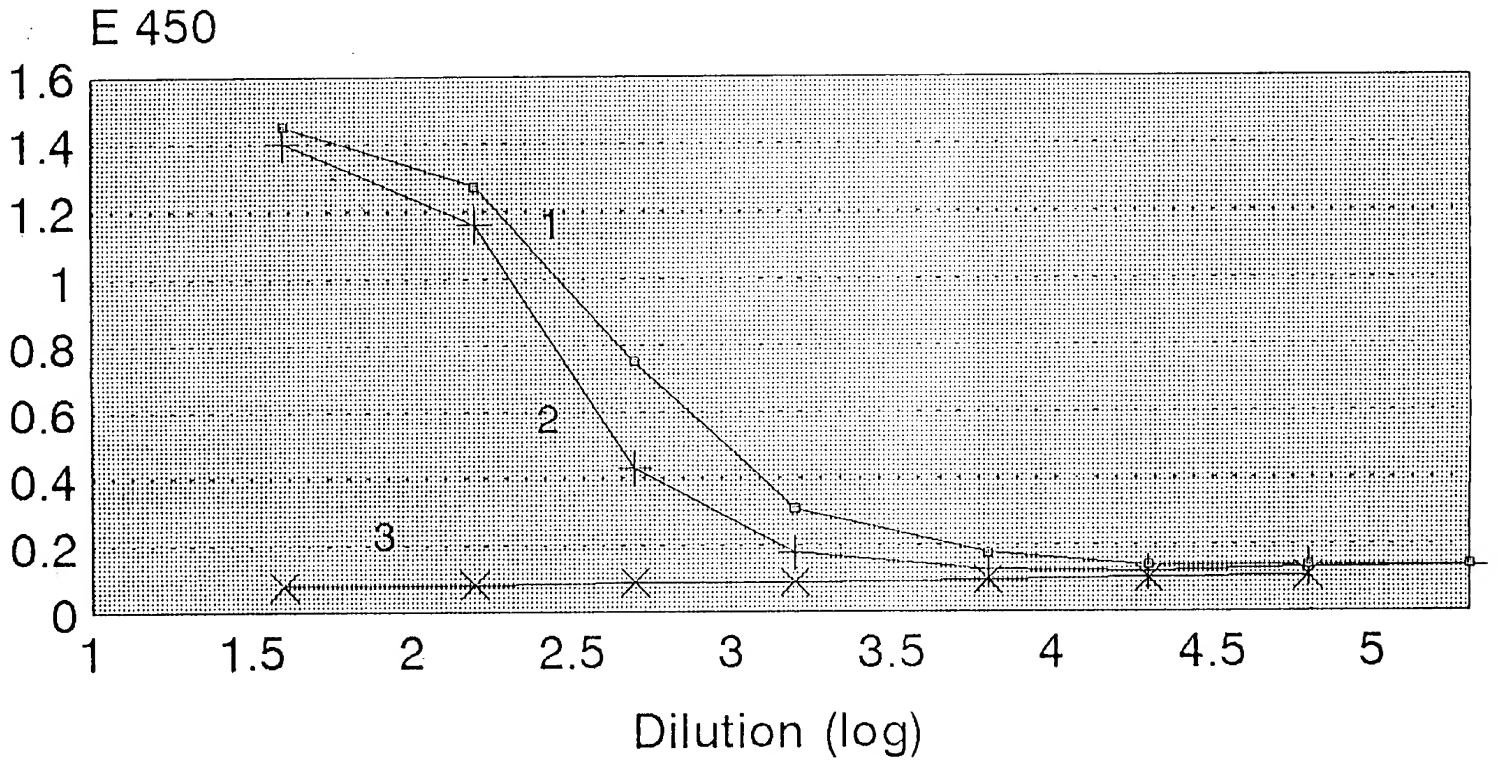


FIGURE 6.

FIG.7 Antigenicity of serum after immunisation of mice with MBP-BoNT/F(848-1278) recombinant protein

Antigen: BoNT/F, 13 ng/well

Sera S-2 (1) and S-3 (2) were after second and third boosts



Serum S-2, S-3 as well as non-immune serum were first diluted 1:50 and 1:3 at each next step

(3): Non-immune sera